植物研究雑誌
J. Jpn. Bot.
79: 255–261 (2004)

Phylogenetic Analysis of the Tetrasporalean Genus *Asterococcus* (Chlorophyceae) Based on 18S Ribosomal RNA Gene Sequences

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(Received on October 30, 2003)

Nucleotide sequences (1642 bp) from 18S ribosomal RNA genes were analyzed for 100 strains of the clockwise (CW) group of Chlorophyceae to deduce the phylogenetic position of the immotile colonial genus *Asterococcus* Scherffel, which is classified in the Palmellopsidaceae of Tetrasporales. We found that the genus *Asterococcus* and two unicellular, volvocalean genera, *Lobochlamys* Pröschold & al. and *Oogamochlamys* Pröschold & al., formed a robust monophyletic group, which was separated from two tetrasporalean clades, one composed of *Tetraspora* Link and *Paulschulzia* Skuja and the other containing the other palmellopsidacean genus *Chlamydocapsa* Fott. Therefore, the Tetrasporales in the CW group is clearly polyphyletic and taxonomic revision of the order and the Palmellopsidaceae is needed.

Key words: 18S rRNA gene, *Asterococcus*, Palmellopsidaceae, phylogeny, Tetrasporales.

Asterococcus Scherffel (1908) is a colonial green algal genus that is characterized by an asteroid chloroplast in the cell and swollen gelatinous layers surrounding the immotile colony (e. g., Ettl and Gärtner 1988). This genus is generally assigned to the Tetrasporales (e. g., Lemmermann 1915, Tiffany 1934, Smith 1950, Ettl 1964, Fott 1971, Ettl and Gärtner 1988). However, the systematic position of *Asterococcus* at the family level varies according to the author. Lemmermann (1915) classified *Asterococcus* in the Palmellaceae. Korshikov (1953) considered this genus a member of the dendroid family Chlorangiellaceae because his alga produced dendroid, gelatinous colonies. Fott (1971) distinguished *Asterococcus* from other members of the Tetrasporales and established a new family, the Asterococcaceae. Recently, Ettl and Gärtner (1988) included *Asterococcus* in the family Palmellopsidaceae, because cells of this genus have contractile vacuoles and lack pseudoflagella in the non-attached vegetative colony.

Patricia et al. (2000) revealed that *Asterococcus* belongs to the clockwise (CW) group of Chlorophyceae, based on the CW orientation of the flagellar apparatus in *A. superbus* (Cienkowski) Scherffel. Nakazawa et al. (2004) carried out a taxonomic study of *Asterococcus* based on the comparative morphology of many strains and molecular phylogenetic analyses of the Rubisco large subunit (*rbc*L) gene sequences. However, the phylogenetic position of this genus within the CW group was ambiguous, possibly because of the limited numbers of operational taxonomic units (OTUs) analyzed (Naka-

zawa et al. 2004). Therefore, sequence data of *Asterococcus* are needed for genes that have been determined in many taxa in the CW group. Although Pröschold et al. (2001) studied the phylogeny of 95 strains of the CW group based on 18S ribosomal (r) RNA genes, *Asterococcus* sequences were not analyzed. Therefore we evaluated the phylogenetic position of the genus *Asterococcus* based on 18S rRNA sequences. Our results suggest that *Asterococcus* is separated from other members of the Tetrasporales within the CW group.

Material and Methods

Two strains of *Asterococcus* were selected as representatives of the genus since they have different colonial forms and belong to the two subgroups constituting the monophyletic *Asterococcus* group in the *rbc*L gene phylogeny (Nakazawa et al. 2004). They were *A. superbus* IAM C-299 with a spherical colony and *A. korschikoffii* Ettl ACOI 326 with a dendroid colony (Nakazawa et al. 2004) (Table 1).

The methods for DNA extraction and direct sequencing of polymerase chain reaction (PCR) products were the same as those of a previous study (Nakazawa et al. 2004), except for the primers used for PCR and sequencing of 18S rRNA genes (Table 2).

Ninety five OTUs in the CW group examined in this study were the same as those of Pröschold et al. (2001), and five additional CW OTUs are listed in Table 1. Seven OTUs of the directly opposed (DO) group (Fig. 1) were selected from Pröschold et al. (2001). The alignment of the 18S rRNA genes from the 95 CW and seven DO OTUs was extracted from that of Pröschold et al. (2001) and the five additional OTUs (Table 1) were realigned using Clustal X (Thompson et al. 1997).

The alignment (1642 bp; see Pröschold et al. 2001) was subjected to unweighted maximum parsimony (MP) analysis, including bootstrap analysis (Felsenstein 1985) with 100 replications of a full heuristic search based on the nearest-neighbor interchange (NNI) branch-swapping algorithm, using PAUP 4.0b10 (Swofford 2002). Following the guidelines for topology construction with distance methods (Nei and Kumar 2000), we selected Jukes-Cantor distances (Jukes and Cantor 1969) to construct neighbor-joining (NJ) trees. From the alignment used for the MP analysis, a Jukes-Cantor distance matrix was calculated (Jukes and Cantor 1969) using PAUP 4.0b10. With the NJ algorithm (Saitou and Nei 1987), a

Table 1. Additional OTUs of CW group (Pröschold et al. 2001) used for phylogenetic analysis using the18S rRNA genes

Species	Strain designation	Origin of 18S sequence data	DDBJ/EMBL/GenBank accession number
Asterococcus superbus	IAM[1] C-299	The present study	AB175836
Asterococcus korschikoffii	ACOI[2] 326	The present study	AB175837
Characiochloris acuminata	UTEX[3] 2095	Buchheim et al. (2002)	AF395435
Hafniomonas montana	NIES[4]-257	Nozaki et al. (2003)	AB101517
Hafniomonas montana	NIES-656	Nozaki et al. (2003)	AB101518

[1]IAM Culture Collection at the University of Tokyo (Sugiyama et al. 1998).
[2]The Culture Collection of Algae at the Department of Botany, University of Coimbra (Santos and Mesquita 1986, Santos 1988, Santos et al. 1993).
[3]Culture Collection of Algae at the University of Texas at Austin (Starr and Zeikus 1993).
[4]Microbial Culture Collection at the National Institute for Environmental Studies (Watanabe et al. 2000).

Table 2. Primers used for amplifications and sequencing of the18S rRNA genes

Designation	Positions[1]	Sequence (5'–3')
18S-FA	1–21	AACCTGGTTGATCCTGCCAGT
18S-RD	570–550[2]	GCTGGCACCAGACTTGCCCTC
18S-FG	557–577	AGTCTGGTGCCAGCAGCCGCG
18S-FE	1112–1132	GGGAGTATGGTCGCAAGGCTG
18S-RF	1202–1182[2]	CCCGTGTTGAGTCAAATTAAG
18S-RB	1799–1774[2]	TGATCCTTCTGCAGGTTCACCTAC

[1]Coordinate number from the *Chlorella vulgaris* Beijerink 18S rRNA gene (Huss et al. 1990).
[2]Reverse primer.

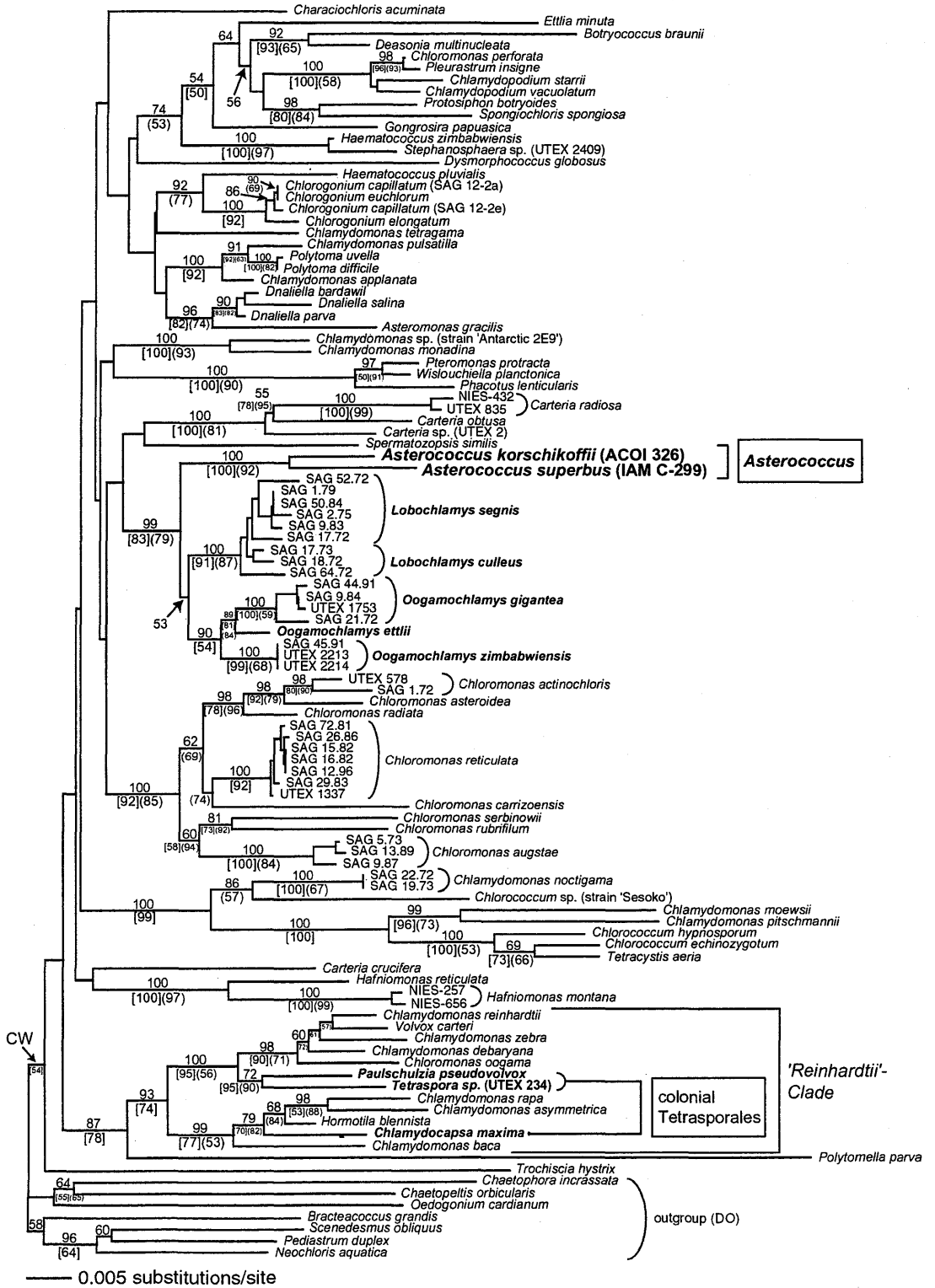
phylogenetic tree was constructed, also using PAUP 4.0b10. The robustness of the resulting lineages was tested by bootstrap analysis with 1000 replications using PAUP 4.0b10. Using the same alignment data, a maximum likelihood (ML) analysis was carried out using PUZZLE in PAUP 4.0b10, with the HKY model (Hasegawa et al. 1985) to estimate quartet puzzling support values, which have the same practical meaning as bootstrap values (Strimmer and von Haeseler 1996) for internal branches of the phylogenetic tree with 1000 puzzling steps (comparable to the number of bootstrap replicates). In these phylogenetic analyses, the seven species in the DO group (Fig. 1) were designated the outgroup since the DO group is sister to the CW group containing volvocalean and biflagellate tetrasporalean algae (Booton et al. 1998).

Results

The NJ tree of the 18S rRNA sequences is shown in Fig. 1, and branches with 50 % or greater bootstrap/QPS values in the NJ, MP and/or ML are shown. A robust monophyletic group consisting of the genus *Asterococcus* (*A. superbus* and *A. korschikoffii*) was resolved with high bootstrap/QPS values (92–100 %) using the NJ, MP, and ML methods. This genus was distant from the '*Reinhardtii*'-Clade (Pröschold et al. 2001), which included two separate lineages of other tetrasporalean algae, one containing a palmellopsidacean species [*Chlamydocapsa maxima* (Mainx) Ettl & Gärtner] and the other composed of two colonial genera in the Tetrasporaceae (*Tetraspora* Link and *Paulschulzia* Skuja). By contrast, the genus *Asterococcus* constituted a robust monophyletic group with two unicellular volvocalean genera *Oogamochlamys* Pröschold & al. (2001) and *Lobochlamys* Pröschold & al. (2001) with high bootstrap/QPS values (79–99 %) with the NJ, MP, and ML methods. Within this group, the MP and ML analyses suggested that *Asterococcus* and *Oogamochlamys* formed a clade with 71–75 % bootstrap/QPS values (not shown), whereas the NJ analysis did not support this clade.

Discussion

Tetrasporales is traditionally characterized as having a gelatinous structure (gelatinous matrix and/or pseudoflagella) in the vegetative phase in which cells can be converted into reproductive cells without preceding cell divisions (e. g., Ettl and Gärtner 1988). However, the validity of this order has been doubted because no common characteristics clearly distinguish the Tetrasporales from the volvocalean and/or chlorococcalean green algae (e. g., van den Hoek et al. 1995). Based on the 18S rRNA gene phylogeny, Booton et al. (1998) demonstrated that the Tetra-


Characiochloris acuminata
Ettlia minuta
Botryococcus braunii
Deasonia multinucleata
Chloromonas perforata
Pleurastrum insigne
Chlamydopodium starrii
Chlamydopodium vacuolatum
Protosiphon botryoides
Spongiochloris spongiosa
Gongrosira papuasica
Haematococcus zimbabwiensis
Stephanosphaera sp. (UTEX 2409)
Dysmorphococcus globosus
Haematococcus pluvialis
Chlorogonium capillatum (SAG 12-2a)
Chlorogonium euchlorum
Chlorogonium capillatum (SAG 12-2e)
Chlorogonium elongatum
Chlamydomonas tetragama
Chlamydomonas pulsatilla
Polytoma uvella
Polytoma difficile
Chlamydomonas applanata
Dnaliella bardawil
Dnaliella salina
Dnaliella parva
Asteromonas gracilis
Chlamydomonas sp. (strain 'Antarctic 2E9')
Chlamydomonas monadina
Pteromonas protracta
Wislouchiella planctonica
Phacotus lenticularis
NIES-432
UTEX 835
Carteria radiosa
Carteria obtusa
Carteria sp. (UTEX 2)
Spermatozopsis similis
Asterococcus korschikoffii (ACOI 326)
Asterococcus superbus (IAM C-299)
Asterococcus
SAG 52.72
SAG 1.79
SAG 50.84
SAG 2.75
SAG 9.83
SAG 17.72
Lobochlamys segnis
SAG 17.73
SAG 18.72
SAG 64.72
Lobochlamys culleus
SAG 44.91
SAG 9.84
UTEX 1753
SAG 21.72
Oogamochlamys gigantea
Oogamochlamys ettlii
SAG 45.91
UTEX 2213
UTEX 2214
Oogamochlamys zimbabwiensis
UTEX 578
SAG 1.72
Chloromonas actinochloris
Chloromonas asteroidea
Chloromonas radiata
SAG 72.81
SAG 26.86
SAG 15.82
SAG 16.82
SAG 12.96
SAG 29.83
UTEX 1337
Chloromonas reticulata
Chloromonas carrizoensis
Chloromonas serbinowii
Chloromonas rubrifilum
SAG 5.73
SAG 13.89
SAG 9.87
Chloromonas augstae
SAG 22.72
SAG 19.73
Chlamydomonas noctigama
Chlorococcum sp. (strain 'Sesoko')
Chlamydomonas moewsii
Chlamydomonas pitschmannii
Chlorococcum hypnosporum
Chlorococcum echinozygotum
Tetracystis aeria
Carteria crucifera
Hafniomonas reticulata
NIES-257
NIES-656
Hafniomonas montana
Chlamydomonas reinhardtii
Volvox carteri
Chlamydomonas zebra
Chlamydomonas debaryana
Chloromonas oogama
Paulschulzia pseudovolvox
Tetraspora sp. (UTEX 234)
Chlamydomonas rapa
Chlamydomonas asymmetrica
Hormotila blennista
Chlamydocapsa maxima
Chlamydomonas baca
'Reinhardtii'-Clade
colonial Tetrasporales
Polytomella parva
Trochiscia hystrix
Chaetophora incrassata
Chaetopeltis orbicularis
Oedogonium cardianum
Bracteacoccus grandis
Scenedesmus obliquus
Pediastrum duplex
Neochloris aquatica
outgroup (DO)
CW
0.005 substitutions/site

sporales is polyphyletic, composed of a biflagellate/CW group and a quadriflagellate/DO group within the Chlorophyceae. We clearly resolved three separate lineages of the tetrasporalean algae within the CW group (Fig. 1), and concluded that Tetrasporales is polyphyletic even within the CW group. Therefore, an immotile vegetative phase with gelatinous structures and the direct formation of the reproductive cells characterizing the Tetrasporales likely evolved in multiple phylogenetic positions in the CW group, and the taxonomic separation of Tetrasporales from Volvocales/Chlorococcales does not represent the natural phylogenetic relationships within the green algae.

Based on this study, *Asterococcus* and two biflagellate unicellular genera *Oogamochlamys* and *Lobochlamys* (Volvocales) form a robust monophyletic group within the CW group. However, no morphological features seem to characterize *Oogamochlamys/Lobochlamys* and *Asterococcus* at the light microscopic level (Pröschold et al. 2001, Nakazawa et al. 2004). Although the mucilage layer around the cell of *Lobochlamys* (Pröschold et al. 2001) seems to be homologous to the colonial gelatinous layers of *Asterococcus*, such a layer is also recognized in another unicellular genus, *Vitreochlamys* in the CW group (e. g., Nakazawa et al. 2001). Patricia et al. (2000) and Nakazawa et al. (2004) reported details of the cell structure of *Asterococcus* using transmission electron microscopy. However, there are no morphological studies of *Oogamochlamys* and *Lobochlamys* based on ultrastructural observations. Further electron microscopic studies of these two genera may resolve ultrastructural features characterizing *Asterococcus* and *Oogamochlamys/Lobochlamys* and contribute to reconstruction of a natural taxonomic system of the CW green algae traditionally classified as members of the Tetrasporales.

We are grateful to Dr. T. Pröschold for providing the alignment of the 18S rRNA genes. This study was supported in part by a grant from the Institute for Fermentation (to HN).

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Fig. 1. Distance tree based on aligned nucleotide sequences for 1642 bp in the 18S rRNA genes for two strains of *Asterococcus* and 98 other strains of CW group and seven DO algae. The tree was derived with the neighbor-joining method (Saitou and Nei 1987) based on the Jukes-Cantor distance (Jukes and Cantor 1969) indicated by the scale bar below the tree. Numbers above branches are the bootstrap values (50 % or more) based on 1000 replications. Numbers in brackets below the branches are bootstrap values resolved in the majority-rule (50 %) consensus tree of a bootstrap analysis based on 100 replications of most parsimonious analysis by PAUP 4.0b10. Numbers in parentheses below the branches are quartet puzzling support (QPS) values (50 % or more) of a maximum likelihood analysis with 1000 puzzling steps. Bootstrap/QPS values at distal branches within *Oogamochlamys*, *Lobochlamys* and *Chloromonas* are not shown. Designations of species names and the alignment of 18S rRNA genes are almost based on those of Pröschold et al. (2001). ACOI: The Culture Collection of Algae at the Department of Botany, University of Coimbra (Santos and Mesquita 1986, Santos 1988, Santos et al. 1993). IAM: IAM Culture Collection at the University of Tokyo (Sugiyama et al. 1998). NIES: Microbial Culture Collection at the National Institute for Environmental Studies (Watanabe et al. 2000). SAG: Sammlung von Algenkulturen at the University of Göttingen (Schlösser 1994). *UTEX*: Culture Collection of Algae at the University of Texas at Austin (Starr and Zeikus 1993).

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中沢　敦，野崎久義：18S rRNA 遺伝子配列を用いたヨツメモ目 *Asterococcus* 属（緑藻綱）の分子系統解析

Asterococcus Scherffel は放射状の葉緑体と細胞群体を包む膨潤した寒天状基質層により特徴付けられる不動性微細藻であり，緑藻綱，ヨツメモ目，パルメロプシス科に分類されている．筆者らは本属の緑藻綱の CW グループ（鞭毛基部の配置が時計回り型のボルボックス目，クロロコックム目の一部，2 鞭毛性ヨツメモ目を含む一群）内における系統的位置を明らかにするため，18S rRNA 遺伝子1642塩基対を用いた系統解析を行った．その結果，*Asterococcus* は遊泳性の単細胞性ボルボックス目の *Lobochlamys* 及び *Oogamochlamys* と高いブートストラップ確率で単系統をなし，*Tetraspora* を含むヨツメモ目のクレード及び，パルメロプシス科に属する *Chlamydocapsa maxima* (Mainx) Ettl & Gärtner と分離することが示された．

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